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Cadherin-11 modulates the terminal differentiation and fusion of human trophoblastic cells in vitro

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Abstract

E-cadherin and cadherin-11 are two members of the cadherin gene family of cell adhesion molecules that are differentially expressed during the aggregation, differentiation, and fusion of trophoblasts isolated from the human term placenta. E-cadherin expression is highest in cytotrophoblasts and decreases as these mononucleate cells undergo terminal differentiation and fusion. In contrast, cadherin-11 expression increases during the formation of multinucleated syncytium in these primary cultures. To define the role(s) of cadherin-11 in this developmental process, we examined the effects of ectopic cadherin-11 expression on the differentiation and fusion of JEG-3 choriocarcinoma cells, a mononucleate trophoblastic cell line. Cadherin-11 expression, but not the ectopic expression of the related cadherin subtype, cadherin-6, resulted in the formation of multinucleated syncytium in the transfected JEG-3 cell cultures. Multinucleated syncytium formation in the JEG-3 cells transfected with cadherin-11 was associated with a reduction in E-cadherin, α-, β-, γ-catenin, and $p120^{ctn}$ expression. Cadherin-11 also reduced cell proliferation and increased the levels of the mRNA transcript encoding the beta subunit of human chorionic gonadotropin, a biochemical marker of trophoblast differentiation, in these cultures. Furthermore, primary cytotrophoblasts cultured in the presence of antisense oligonucleotides specific for cadherin-11 maintained E-cadherin expression and did not undergo terminal differentiation and fusion with time in culture. Collectively, these observations demonstrate that cadherin-11 contributes to the morphological and functional differentiation of cultured mononucleate trophoblastic cells in a highly specific manner.

Keywords: Cadherin-11; Cell adhesion molecules; Catenins; Human placenta; Trophoblast differentiation

Introduction

The cadherins are a gene superfamily of integral membrane glycoproteins that mediate calcium-dependent homophilic cell adhesion (Takeichi, 1995; Suzuki, 1996). This gene superfamily has been divided into at least two distinct subfamilies: type 1 and type 2 classical cadherins. The type 1 cadherins include R-cadherin, also known as cadherin-4, and the three originally identified cadherins, E-cadherin (E-cad), N-cadherin (N-cad), and P-cadherin, whereas human cadherin-6, -7, -8, -9, -10, -11, -12, -14, -18, -19, and -20 have been assigned to the type 2 cadherin subfamily (Nollet et al., 2000; Yagi and Takeichi, 2000).

The type 1 and type 2 classical cadherins share common structural features (Suzuki et al., 1991; Tanihara et al., 1994). Although the overall amino acid sequence homology between these two subfamilies is low, the cytoplasmic domains of the type 1 and type 2 cadherins are highly conserved (Suzuki et al., 1991). These domains interact with a group of proteins, known as the catenins. β-Catenin and γ-catenin (also known as plakoglobin) interact with the cadherins in a mutually exclusive manner (Butz and Kemler, 1994; Nathke et al., 1994). α-Catenin, in turn, links both cadherin-catenin complexes to the actin-based cytoskeleton by direct interaction (Rimm et al., 1995) or via α-actinin (Knudsen et al., 1995). $p120^{ctn}$, a substrate for the Src tyrosine kinases, has also been shown to interact with the cadherin cytoplasmic domain at a different site from that of β- or γ-catenin (Reynolds et al., 1989, 1992; Ohkubo and Ozawa, 1999). The binding of the catenins to the cadherin cytoplasmic tail is believed to alter the strength of cadherin-

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based adhesion complexes (Brieher et al., 1996; Ozawa and Kemler, 1998).

The cadherins are key morphoregulators (Takeichi, 1995, Tepass et al., 2000). The spatiotemporal expression of both the type 1 and type 2 classical cadherins is tightly regulated during embryonic development. For example, the differential expression of E-cad and cadherin-11 (cad-11) has been associated with the formation of mesodermal cell layers in the rodent embryo (Hoffmann and Balling, 1995; Simonneau et al., 1995). The regulated expression of the type 1 cadherins has also been shown to govern the developmental fate of cells. In particular, the transfection of a full-length E-cad cDNA into embryonic stem cells lacking endogenous cadherin expression results in epithelial cell differentiation, whereas N-cad expression promotes the formation of cartilage and neuroepithelial cell structures (Larue et al., 1996). To date, the ability of the type 2 cadherins to modulate cellular differentiation remains poorly characterized.

Villous cytotrophoblasts of the human placenta undergo cellular differentiation and fusion to form the syncytial trophoblast, a multinucleated cell that contributes to the majority of placental transport, immunoregulatory, and endocrine functions during pregnancy (Richart, 1961; Kliman et al., 1986). We have determined that E-cad and cad-11 are differentially expressed during the terminal differentiation and fusion of mononucleate villous cytotrophoblasts isolated from the human term placenta (MacCalman et al., 1996). In particular, E-cad expression levels are high in freshly isolated cytotrophoblasts and decrease as the cells undergo aggregation and fusion to form multinucleated syncytium with time in culture. The loss of E-cad expression during this cellular event was concomitant with a marked increase in the expression levels of cad-11. The formation of multinucleated syncytium in these cell cultures also correlates with a marked reduction in the expression levels of α-, β-, γ-catenin and p120^{ctn} (Getsios et al., 2001). In contrast, E-cad and these four catenin subtypes, but not cad-11, are readily detectable in nonfusing JEG-3 choriocarcinoma cells. Collectively, these observations have led us to hypothesize that cad-11 mediates the formation of multinucleated syncytium from mononucleate trophoblastic cells in vitro.

Here, we report that the exogenous expression of cad-11, but not cadherin-6 (cad-6), in mononucleate JEG-3 cells is capable of promoting the morphological and functional differentiation of these trophoblastic cells. The formation of multinucleated syncytium in these transiently transfected cells correlated with a reduction in the expression levels of E-cad and α-, β-, γ-catenin and p120^{ctn}. Antisense oligonucleotides specific for cad-11 inhibited the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta. Collectively, we believe that these observations add to our understanding of the adhesive mechanisms underlying the formation and organization of the human placenta.

Materials and methods

Tissues

Term placentae were obtained from women undergoing Caesarian section. Follicular aspirates were collected from the ovaries of patients undergoing in vitro fertilization and embryo transfer. Research using these human tissues was approved by the Committee for Ethical Review of Research Involving Human Subjects, University of British Columbia. All subjects provided informed written consent.

Cell isolation and culture

Granulosa-lutein cells were isolated from human ovarian follicles according to the methods described by Golos and Strauss (1987). Briefly, the follicular aspirates were centrifuged on a Ficoll-Paque gradient (Pharmacia Biotech Inc., Baie D'Urfe, PQ) at 1000*g* for 10 min at room temperature. The granulosa-lutein cells were collected from the gradient interface before being washed, resuspended, and plated in Dulbecco's modified Eagle's medium (Gibco BRL, Burlington, ON) containing 25 mM glucose, 25 mM Hepes, and 50 μg/ml gentamicin and supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Labs Inc., Logan, UT).

Villous cytotrophoblasts were isolated from human term placental tissues as previously described by Kliman et al. (1986). This method, which utilizes serial trypsin–DNase I digestions, yields a highly enriched preparation of mononucleate cytotrophoblasts. Following isolation, the cells were resuspended and plated in the culture medium described above. JEG-3 choriocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in the culture medium described above.

Expression vectors

Full-length cad-11 or cad-6 cDNAs (gifts from ICOS Corp., Bothell, WA) were subcloned into the *Not*I restriction site of the mammalian expression vector, pCMV/SPORT1 (Gibco BRL), using standard molecular biology techniques. Clones containing the cad-11 or cad-6 cDNA in the forward (pCAD-11 or pCAD-6) or reverse orientation (pRCAD-11 or pRCAD-6) were identified by DNA sequence analysis. A pCMV/SPORT1 expression vector containing the β-galactosidase gene (p*lac*Z; Gibco BRL) was used to determine transfection efficiency, whereas pRCAD-6 and pRCAD-11 served as controls for these studies.

The ability of exogenous cad-11 expression to modulate the morphological and functional differentiation of JEG-3 choriocarcinoma cells was then examined. JEG-3 cells (2.5×10^5 cells) were seeded on glass coverslips (2×2 cm) and cultured for 12 h. The cells were then transfected with

pCAD-11, pRCAD-11, or p*lac*Z (1.0 μg/ml) by using the Fugene 6 transfection reagent (Roche Diagnostics, Laval, PQ) and cultured for a further 0, 12, 24, or 36 h.

Antisense oligonucleotides

Oligonucleotide sequences (18 mers, 50% AT/GC content) were selected from the full-length human cad-11 cDNA (GenBank Accession No. L34056.1) by using the Primer3 program (Whitehead Institute, Cambridge, MA). DNA sequences located near the 5′ end of the cad-11 cDNA were compared with the human sequence databases of EMBL and GenBank. Two sequences were identified (5′-GGCGGCTTGTAAACAGTA-3′ and 5′-CACGAAGAACTGGTTCCA-3′), corresponding to bp 168–185 and bp 324–341 of the cad-11 cDNA, respectively. Phosphorothioate-conjugated antisense oligonucleotides (OB-1 and OB-2) complementary to these DNA sequences and the corresponding sense oligonucleotides (OB-3 and OB-4) were prepared (Nucleic Acid and Protein Synthesis Biotechnology Lab, University of British Columbia, Vancouver, BC) and used in these studies.

Granulosa-lutein cells, which express cad-6, cad-11, and N-cad (MacCalman et al., 1997), were cultured in the presence of the antisense oligonucleotides, OB-1 or OB-2. Granulosa-lutein cells (1×10^6 cells) were seeded in 60-mm culture dishes and cultured in the presence of increasing concentrations (0, 1, or 5 μM) of antisense (OB-1 or OB-2) or sense oligonucleotides (OB-3 or OB-4) for a further 24 h. The concentrations of oligonucleotides used in these experiments were selected on the basis of previous studies (Caniggia et al., 1997).

The ability of these antisense oligonucleotides to inhibit the terminal differentiation and fusion of villous cytotrophoblasts isolated from the human term placenta was then examined. Mononucleate cytotrophoblasts (2×10^5 cells) were seeded on glass coverslips (2×2 cm) and cultured in the presence of OB-1 or OB-2 (5 μM) for 24, 48, or 72 h. Cytotrophoblasts cultured in the presence of the sense oligonucleotides, OB-3 or OB-4 (5 μM), served as a control for these studies.

Northern blot analysis

Total RNA was prepared from cultures of granulosa-lutein cells or JEG-3 cells by using the phenol-chloroform method of Chomczynski and Sacchi (1987). The RNA species were resolved by electrophoresis in 1% agarose gels containing 3.7% formaldehyde. Approximately 20 μg of total RNA was loaded in each lane. The fractionated RNA species were then transferred onto charged nylon membranes (Amersham Canada Ltd., Oakville, ON).

The Northern blots were probed with radiolabeled cDNAs specific for human cad-11 (MacCalman et al., 1996), N-cad (MacCalman et al., 1997), cad-6 (Getsios et al., 1998), the beta subunit of human chorionic gonadotropin cDNA (βhCG; Coutifaris et al., 1991), and a radiolabeled synthetic oligonucleotide specific for 18S rRNA (Szyf et al., 1990) as previously described (MacCalman et al., 1992).

Antibodies

Two mouse monoclonal antibodies specific for human cad-11 (113E and 113H) were used in these studies (gifts from ICOS Corp., Bothell, WA). Mouse monoclonal antibodies directed against human E-cad and the four catenin subtypes, α-, β-, γ-catenin, and p120^{ctn}, were purchased from Transduction Labs (Lexington, KY). A goat polyclonal antibody directed against cad-6 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and a rabbit polyclonal antibody directed against desmoplakin (NW6; Angst et al., 1990) was kindly provided by Dr. K.J. Green (Northwestern University, Chicago, IL). A mouse monoclonal antibody directed against 5-bromo-2′-deoxyuridine (BrdU) was purchased from Roche Diagnostics. Nonspecific isotype-matched antibodies purchased from Dako Corp. (Carpenteria, CA) were used as negative controls in these studies.

Western blot analysis

Cultures of JEG-3 cells or villous cytotrophoblasts were washed three times in PBS and incubated in 100 μl of cell lysis buffer (10 mM Tris–HCl, pH 7.5, containing 0.5% Nonidet P-40, 0.5 mM $CaCl_2$, and 1.0 mM PMSF) at 4°C for 30 min on a rocking platform. The cell lysates were centrifuged at 10,000*g* for 20 min, and the supernatants were used for Western blot analysis. The concentration of protein in the cell lysates was determined by using the BCA kit (Pierce Chemicals, Rockford, IL). Western blots containing aliquots (20 μg) of the cell lysates were prepared and immunoblotted as previously described (MacCalman et al., 1996). The Amersham ECL system was used to detect antibody bound to antigen.

Indirect immunofluorescence

Indirect immunofluorescence was performed by using JEG-3 cells that had been plated on glass coverslips and fixed in methanol at −20°C for 2 min. Coverslips were incubated with primary antibodies for 45 min at 37°C. Primary antibodies were detected by using Alexa Fluor conjugated secondary antibodies (Molecular Probes, Eugene, OR). JEG-3 cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO). The coverslips were examined by using a Leica DMR microscope/Orca Hamamatsu system and analyzed with OpenLab software (Improvision, Lexington, MA).

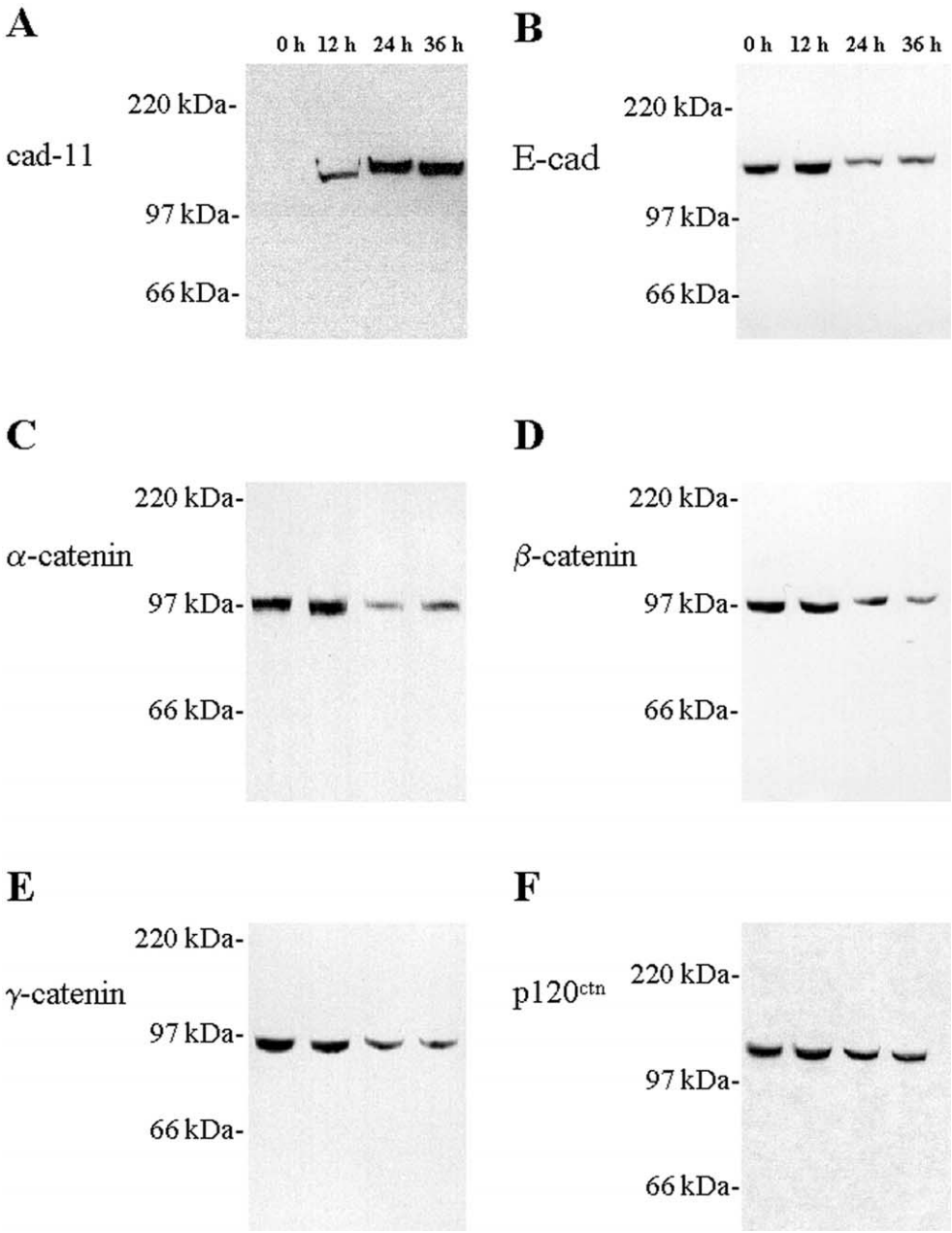


Fig. 1. Cadherin and catenin expression in JEG-3 choriocarcinoma cells transiently transfected with pCAD-11. Autoradiograms of Western blots containing protein extracts (20 μg) prepared from JEG-3 cells transfected with pCAD-11 for 0, 12, 24, or 36 h. Western blot analysis was performed by using mouse monoclonal antibodies directed against human cad-11 (113H), E-cad, α-, β-, γ-catenin, or p120^{ctn} (A–F, respectively). The relative electrophoretic mobilities of the molecular weight markers are shown on the left-hand side of the immunoblots.

Immunocytochemistry

Cultures of JEG-3 cells or villous cytotrophoblasts were fixed in 2% paraformaldehyde/0.2% glutaraldehyde for 15 min at room temperature. Immunocytochemistry was performed according to the methods of Cartun and Pedersen (1989) and included sequential incubations in 10% normal horse serum, primary antiserum at 37°C for 1 h, secondary biotinylated antibody at 37°C for 45 min, streptavidin–biotinylated horseradish peroxidase complex reagent at 37°C for 30 min, and three washes (5 min each) in PBS. The cells were then exposed to chromagen reaction solution (0.035% diaminobenzidine and 0.03% H_2O_2) for 10 min, washed in tap water for 5 min, counterstained in hematoxylin, dehydrated, cleared, and mounted.

BrdU incorporation

A BrdU labeling and detection kit (BrdU labeling and detection kit II; Roche Diagnostics) was used to determine

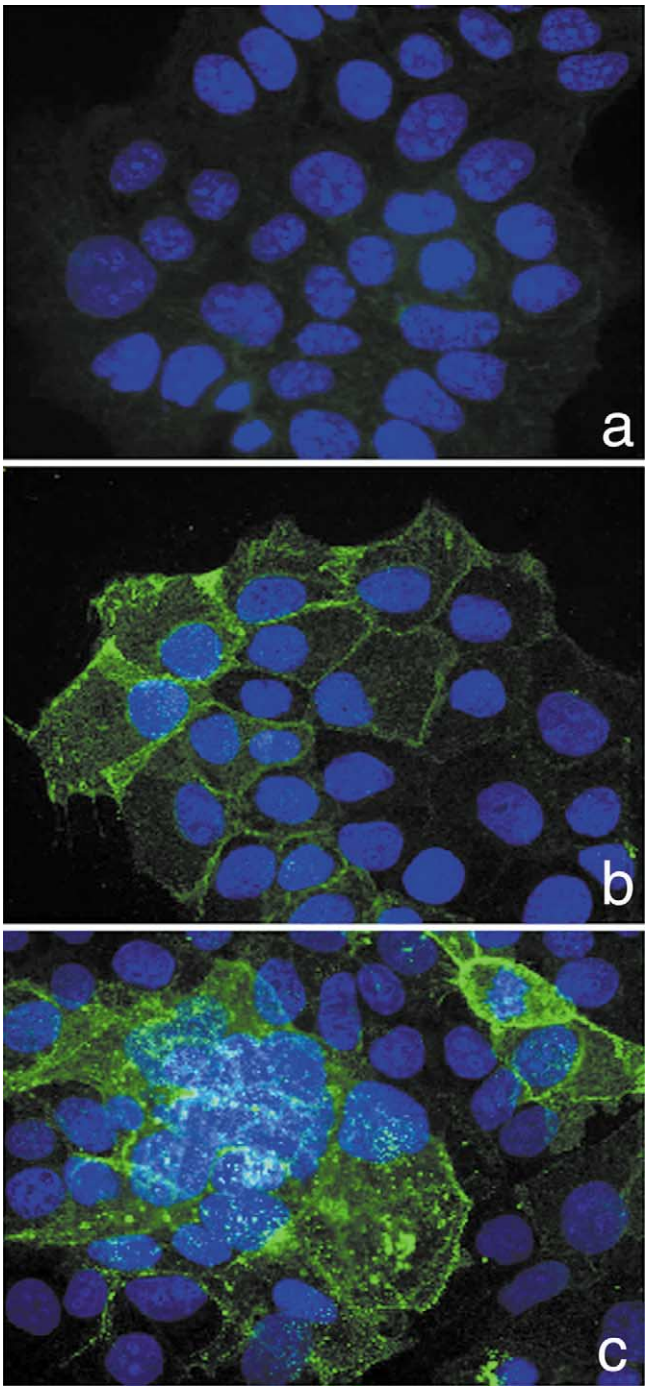


Fig. 2. Immunolocalization of ectopic cad-11 in JEG-3 cells. Photomicrographs of cad-11 expression in JEG-3 cells transfected with pRCAD-11 and cultured for 36 h (a) or pCAD-11 and cultured for 24 or 36 h (b and c, respectively). The cells were fixed and immunostained with a mouse monoclonal antibody directed against human cad-11 (113E). DAPI was used to detect the nuclei in these fixed JEG-3 cell cultures.

the effects of ectopic cad-11 expression on JEG-3 cell proliferation. Briefly, JEG-3 cells were cultured in the presence of BrdU (10 μM) prior to being fixed in 70% ethanol containing 15 mM glycine, pH 2.0, for 20 min at −20°C. BrdU was immunolocalized in these fixed cell cultures by using a mouse monoclonal antibody directed against BrdU.

Results

pCAD-11 promotes the terminal differentiation and fusion of JEG-3 cells: correlation with E-cad, cad-11, and catenin expression levels

β-Galactosidase activity in JEG-3 cells transfected with p*lac*Z demonstrated that >70% of the cells had been transfected with this expression vector construct (data not shown).

We failed to detect cad-11 expression in JEG-3 cells freshly transfected with pCAD-11 using Western blot analysis (Fig. 1), a finding which is consistent with our previous observations in this choriocarcinoma cell line (MacCalman et al., 1996, Getsios et al., 2001). Cad-11 expression was first detected in JEG-3 cells after 12 h of transfection with pCAD-11. There was a marked increase in cad-11 expressed by the JEG-3 cells transfected with pCAD-11 after 24 h. Cad-11 expression levels in these cell cultures remained elevated until the termination of these studies at 36 h. The increase in cad-11 expression in the cells transfected with pCAD-11 was concomitant with a reduction in the expression levels of E-cad and the four catenin subtypes examined in these studies, α-, β-, γ-catenin, and p120^{ctn}. In contrast, E-cad, but not cad-11, was readily detectable in the JEG-3 cells transfected with pRCAD-11 at all of the time points examined in these studies (data not shown). Similarly, the expression levels of α-, β-, γ-catenin, and p120^{ctn} remained relatively constant in JEG-3 cells transfected with pRCAD-11 until the termination of these studies at 36 h.

Cad-11 immunostaining was not observed in JEG-3 cells transfected with pRCAD-11 at any of the time points examined in these studies but was readily detectable in cultures transfected with pCAD-11 for 24 and 36 h (Fig. 2). Cad-11 expression was localized to areas of cell–cell contact 24 h following transfection with pCAD-11 but was distributed diffusely along the surface of the multinucleated syncytium that subsequently formed in these cultures. In contrast, E-cad was immunolocalized to areas of cell–cell contact in JEG-3 cells transfected with pCAD-11 for 12 h (Fig. 3). However, there was a marked and progressive reduction in the intensity of E-cad immunostaining in cultures of JEG-3 cells transfected with pCAD-11 until the termination of these studies at 36 h. A similar cellular distribution was observed for β-catenin (Fig. 3) and the three other catenins examined in these studies, α-, γ-catenin, and p120^{ctn} (data not shown).

To confirm that the mononucleate JEG-3 cells transfected with pCAD-11 were undergoing terminal differentiation and fusion to form multinucleated syncytium, we examined the distribution of desmoplakin in these cell cultures. Desmoplakin is an obligate component of desmo-

somal junctions and has been used as a marker to identify cell boundaries in a wide variety of normal and malignant epithelial cells in vitro, including human trophoblasts (Douglas and King, 1990; Green and Gaudry, 2000). In JEG-3 cells transfected with pCAD-11 for 12 h, desmoplakin was readily detectable at areas of cell–cell contact (Fig. 4). However, there was marked reduction in desmoplakin immunoreactivity in mononucleate JEG-3 cells after 24 h of transfection with pCAD-11. Desmoplakin immunostaining was subsequently localized to the peripheral membrane of multinucleated syncytium that formed in these cell cultures. A similar expression pattern for desmoplakin has been previously detected in cultures of villous cytotrophoblasts undergoing terminal differentiation and fusion in vitro (Douglas and King, 1990). In contrast, intense desmoplakin immunostaining was maintained in JEG-3 cells transfected with pRCAD-11 until the termination of these studies at 36 h.

The morphological effects of pCAD-11 on JEG-3 cells cannot be mimicked by the exogenous expression of cad-6

To determine whether the exogenous expression of a cadherin subtype, other than cad-11, could promote the terminal differentiation and fusion of human trophoblastic cells, JEG-3 cells were transfected with pCAD-11 or pCAD-6 for 36 h. Cad-11 and desmoplakin immunostaining confirmed the presence of multinucleated syncytium in the JEG-3 cell cultures transfected with pCAD-11 (Fig. 5). Although cad-6 was detected on the surface and in areas of cell–cell contact in JEG-3 cells transfected with pCAD-6, the expression of this cadherin subtype did not promote the terminal differentiation and fusion of these trophoblastic cells. Cad-6 expression was not detected in JEG-3 cells transfected with pRCAD-6 (data not shown).

pCAD-11 reduces cellular proliferation and promotes the biochemical differentiation of JEG-3 cells

The terminal differentiation and fusion of human trophoblastic cells in vitro and in vivo is associated with a reduction in cellular proliferation and an increase in the biosynthesis of βhCG (Kliman et al., 1986). In order to determine the effects of ectopic cad-11 expression in JEG-3 cells on these aspects of cellular differentiation, we examined the levels of BrdU incorporation and βhCG mRNA levels in cells transfected with pCAD-11. There was a marked reduction in the level of BrdU incorporation in JEG-3 cells transfected with pCAD-11 for 24 h (Fig. 6). In contrast, the transfection of pRCAD-11 or p*lac*Z did not have a marked effect on DNA synthesis in these cell cultures. Northern blot analysis revealed a single βhCG mRNA transcript (1.1 kb) in all of the total RNA extracts prepared from the JEG-3 cell cultures, consistent with previous observations in this choriocarcinoma cell line (Burnside et al., 1985; Coutifaris et al., 1991). There was a marked increase in the levels of the βhCG mRNA transcript present in JEG-3 cells transfected with pCAD-11 for 36 h (Fig. 6). In contrast, the levels of the mRNA transcript encoding this biochemical marker of trophoblast differentiation remained relatively constant following the transfection of pRCAD-11 or p*lac*Z into these cells.

OB-1 and OB-2 decrease cad-11 mRNA levels in human granulosa-lutein cell cultures

In order to inhibit cad-11 expression in cultured cells, we developed antisense oligonucleotides (OB-1 and OB-2) complementary to the human cad-11 cDNA sequence. The ability of these antisense oligonucleotides to reduce cad-11 mRNA levels was examined in human granulosa-lutein cells, which express three classical cadherin subtypes (N-cad, cad-6, and cad-11) but not E-cad (MacCalman et al., 1997). A single cad-11 mRNA transcript (4.4 kb) was detected in all of the total RNA extracts prepared from the isolated granulosa-lutein cells. This mRNA transcript has been previously observed in human granulosa-lutein, endometrial, and trophoblastic cells (MacCalman et al., 1996, 1997). OB-1 decreased cad-11 mRNA levels in these primary cultures at all of the concentrations examined in these studies (Fig. 7). In contrast, the steady-state levels of the two major N-cad mRNA species (4.3 and 4.0 kb) and the single cad-6 mRNA transcript (4.1 kb) remained relatively constant in granulosa-lutein cells cultured in the presence of this antisense oligonucleotide. The addition of the sense oligonucleotide, OB-3, to the culture medium did not have a marked effect on the levels of the mRNA transcripts encoding the three cadherin subtypes present in human granulosa-lutein cells at any of the concentrations examined in these studies. Similar results were obtained by using granulosa-lutein cells cultured in the presence of our alternative antisense oligonucleotide specific for cad-11, OB-2, or the corresponding sense oligonucleotide, OB-4 (data not shown).

OB-1 inhibits the terminal differentiation and fusion of villous cytotrophoblasts isolated from human term placentae

Western blot analysis demonstrated that villous cytotrophoblasts cultured in the presence of the antisense oligonucleotide, OB-1 (5 μM), failed to upregulate cad-11 expression levels at any of the time points examined in these studies, although we consistently detected lower molecular weight protein species in these cultures 72 h after treatment (Fig. 7). E-cad expression has previously been shown to decrease in isolated human villous cytotrophoblasts in a time-dependent manner (Coutifaris et al., 1991; MacCalman et al., 1996). Instead, the levels of E-cad expression remained relatively constant in the primary cell cultures treated with OB-1 until the termination of these studies at 72 h. There was, however, a marked reduction in E-cad expression levels in villous cytotrophoblasts cultured in the

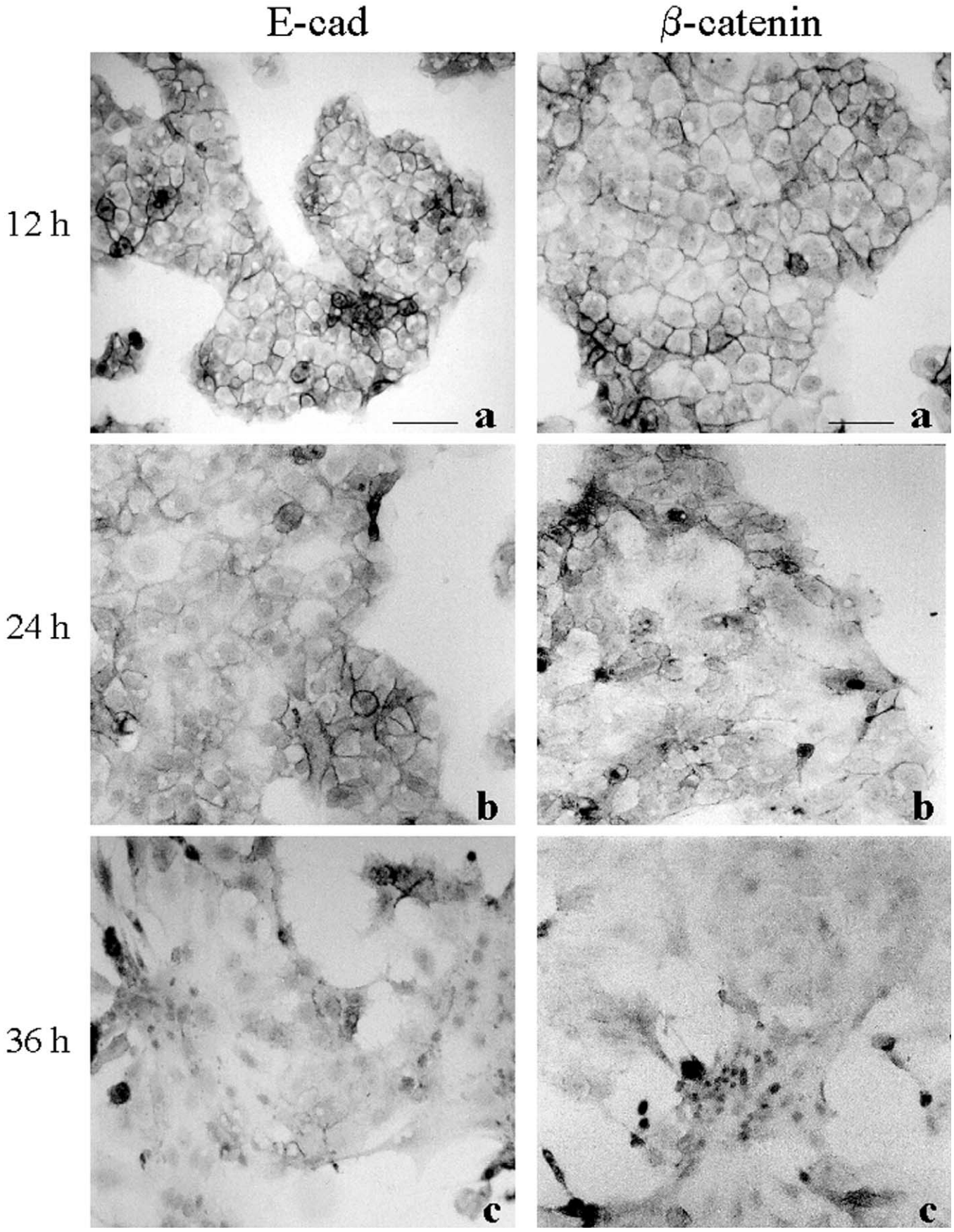


Fig. 3. Immunolocalization of E-cad and β-catenin in JEG-3 cells transfected with pCAD-11. Photomicrographs of E-cad and β-catenin expression in JEG-3 cells transfected with pCAD-11 and cultured for 12, 24, or 36 h (a–c, respectively). The cells were fixed and immunostained with mouse monoclonal antibodies directed against either human E-cad or β-catenin.

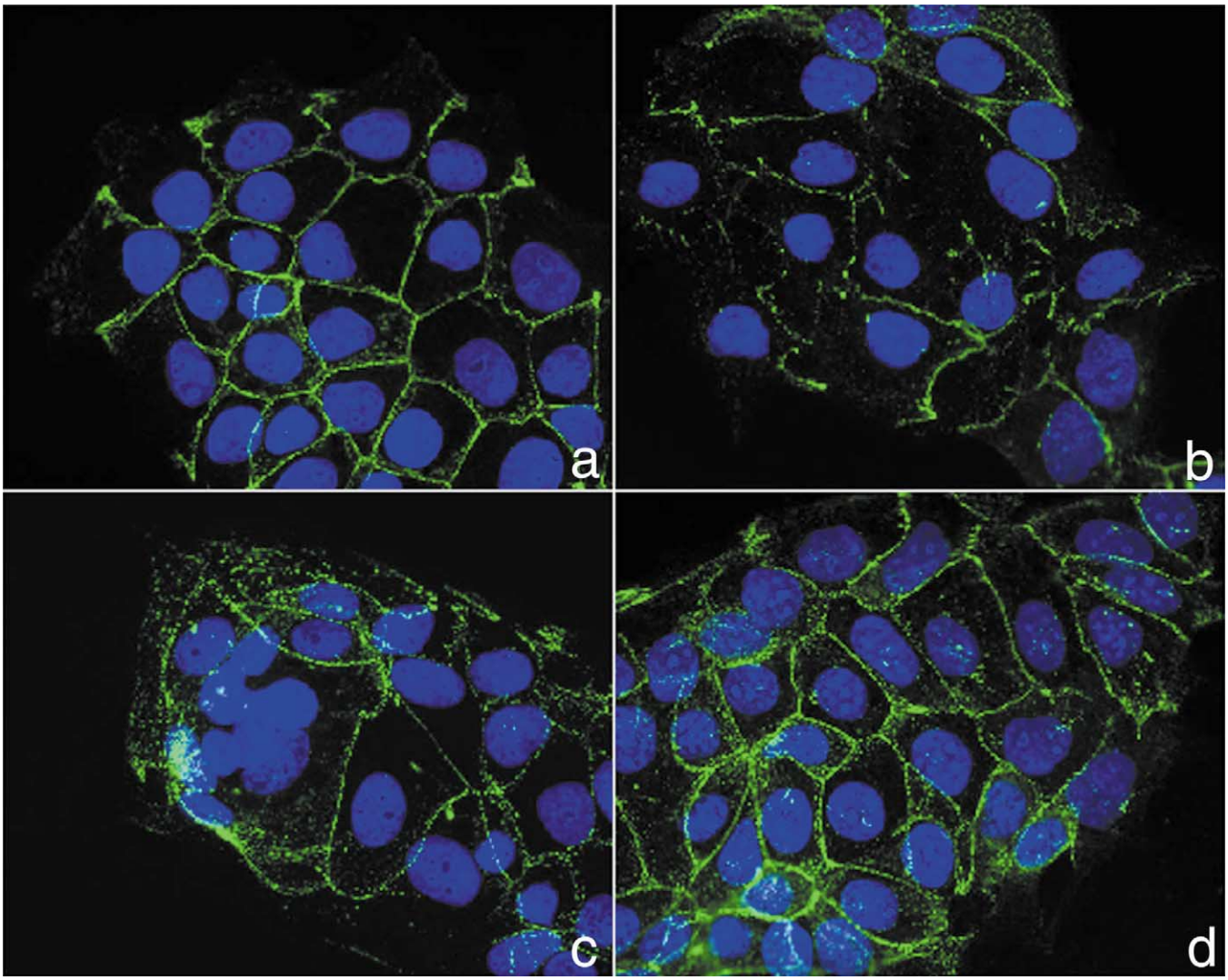


Fig. 4. Immunolocalization of desmoplakin in JEG-3 cells transfected with pCAD-11. Photomicrographs of desmoplakin expression in JEG-3 cells transfected with pCAD-11 and cultured for 12, 24, or 36 h (a–c, respectively). The cells were fixed and immunostained with a rabbit polyclonal antibody directed against desmoplakin (NW6), and the nuclei were visualized by using DAPI. A negative control in which JEG-3 cells were transfected with pRCAD-11 for 36 h is shown in (d).

presence of the sense oligonucleotide, OB-3 (5 μM), for 72 h. In contrast, cad-11 expression levels continued to increase in the trophoblastic cells cultured in the presence of OB-3 until the termination of these studies at 72 h.

Villous cytotrophoblasts cultured in the presence of OB-1 were capable of undergoing aggregation after 72 h of culture (Fig. 7). E-cad expression was immunolocalized to areas of cell–cell contact in these primary cultures, demonstrating that these cellular aggregates were comprised of mononucleate cytotrophoblasts. In contrast, there was a marked reduction in E-cad immunostaining in the multinucleated syncytium that formed in the trophoblastic cells cultured in the presence of OB-3. E-cad expression was, however, maintained in mononucleate cytotrophoblasts that were in direct contact with the multinucleated syncytium in these cultures at 72 h.

Discussion

The differential expression of E-cad and cad-11 during the aggregation and subsequent fusion of villous cytotrophoblasts isolated from human term placentae suggests that these two CAMs have distinct roles in this highly regulated series of membrane-mediated events. Function-perturbing antibodies directed against E-cad disrupted the aggregation of mononucleate villous cytotrophoblasts isolated from the human term placenta, which in turn inhibited the formation of multinucleated syncytium in these cell cultures (Coutifaris et al., 1991). Furthermore, villous cytotrophoblasts cultured in the presence of herbimycin A, an inhibitor of tyrosine kinase activity (Murakima et al., 1988), were capable of upregulating E-cad expression levels but failed to undergo terminal differentiation and fusion (Rebut-

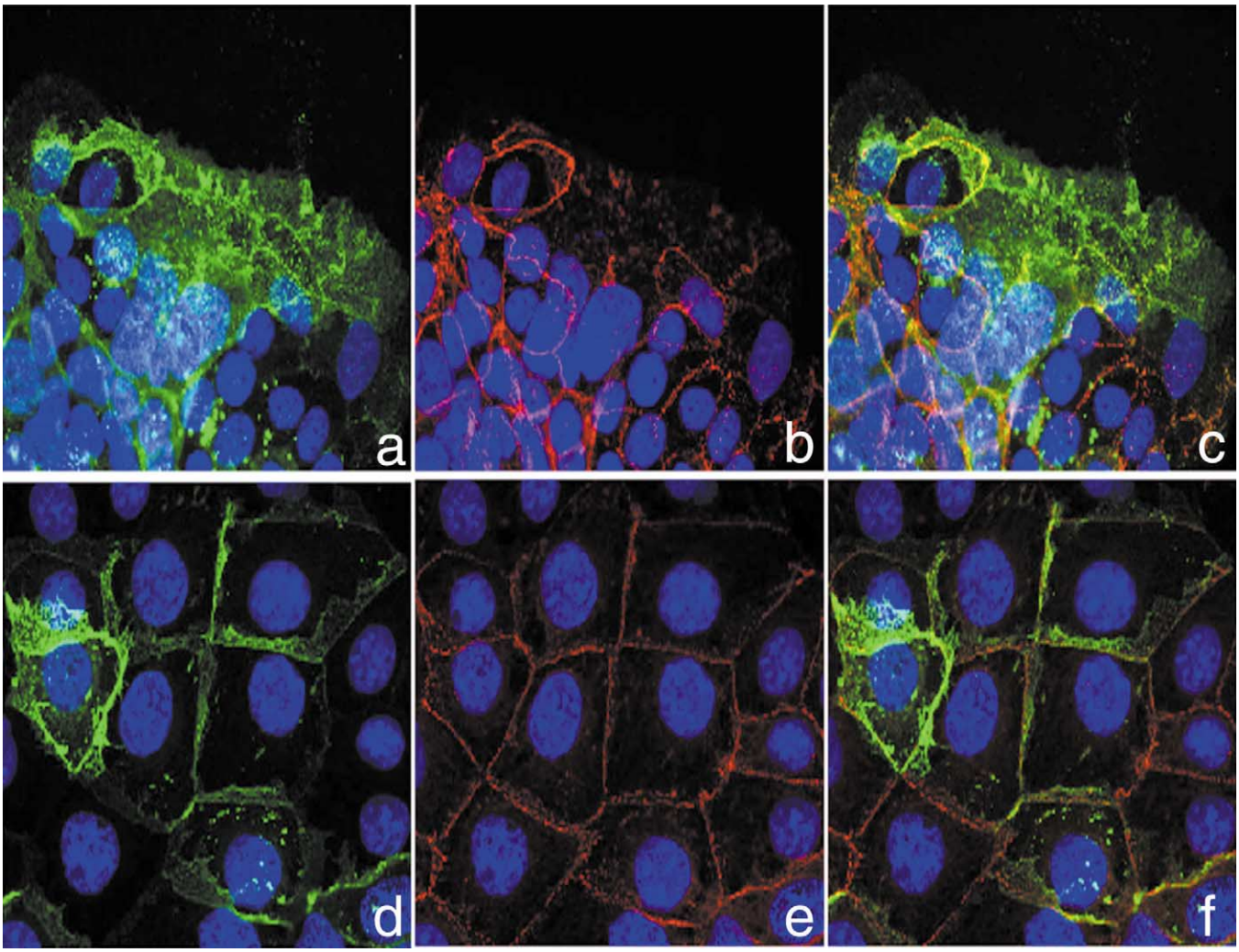


Fig. 5. Immunolocalization of cad-6, cad-11, and desmoplakin in JEG-3 cells transfected with pCAD-6 or pCAD-11. Double-label immunofluorescence was carried out for JEG-3 cells transfected with pCAD-11 (a–c, respectively) or pCAD-6 (d–f, respectively) for 36 h. The ectopic cadherin was detected by using either a mouse monoclonal antibody directed against cad-11 (113E; a) or a goat polyclonal antibody directed against cad-6 (d). A rabbit polyclonal antibody directed against desmoplakin (NW6) was used as a marker for cell–cell borders (b and e). DAPI was used to detect the nuclei in these JEG-3 cell cultures. Dual color overlays are shown on the right (c and f).

Bonneton et al., 1993). Here, we demonstrate that E-cad expression levels are maintained in villous cytotrophoblasts cultured in the presence of antisense oligonucleotides specific for cad-11. These cells were capable of forming cellular aggregates but did not undergo terminal differentiation and fusion to form syncytium with time in culture. Collectively, these observations suggest that E-cad mediates the aggregation of mononucleate trophoblastic cells, whereas cad-11 expression is required for the formation of multinucleated syncytium in these primary cell cultures.

Further support for the hypothesis that cad-11 promotes trophoblast differentiation and fusion in a highly specific manner was obtained from JEG-3 choriocarcinoma cells transfected with full-length cad-11 or cad-6 expression vectors. The ectopic expression of cad-11, but not cad-6, in JEG-3 cells resulted in the formation of multinucleated syncytium from the terminal differentiation and fusion of these mononucleate trophoblastic cells. The formation of multinucleated syncytium in JEG-3 cells transfected with pCAD-11 was associated with the loss of E-cad expression in these cell cultures. Recent studies have demonstrated cell-specific differences in the ability of exogenous cadherins to regulate the expression levels of endogenous cadherin subtypes. For example, the introduction of a full-length N-cad cDNA into oral squamous carcinoma cell lines resulted in a loss of endogenous E-cad expression and induced a fibroblastic phenotype in these cell cultures (Islam et al., 1996). In contrast, ectopic N-cad expression was shown to increase the motility and invasive capacity of breast cancer cell lines without altering the levels of E-cad expression (Nieman et al., 1999; Hazan et al., 2000). To date, the cellular mechanisms that regulate the coordinate expression of transfected full-length cadherin cDNAs with the endog-

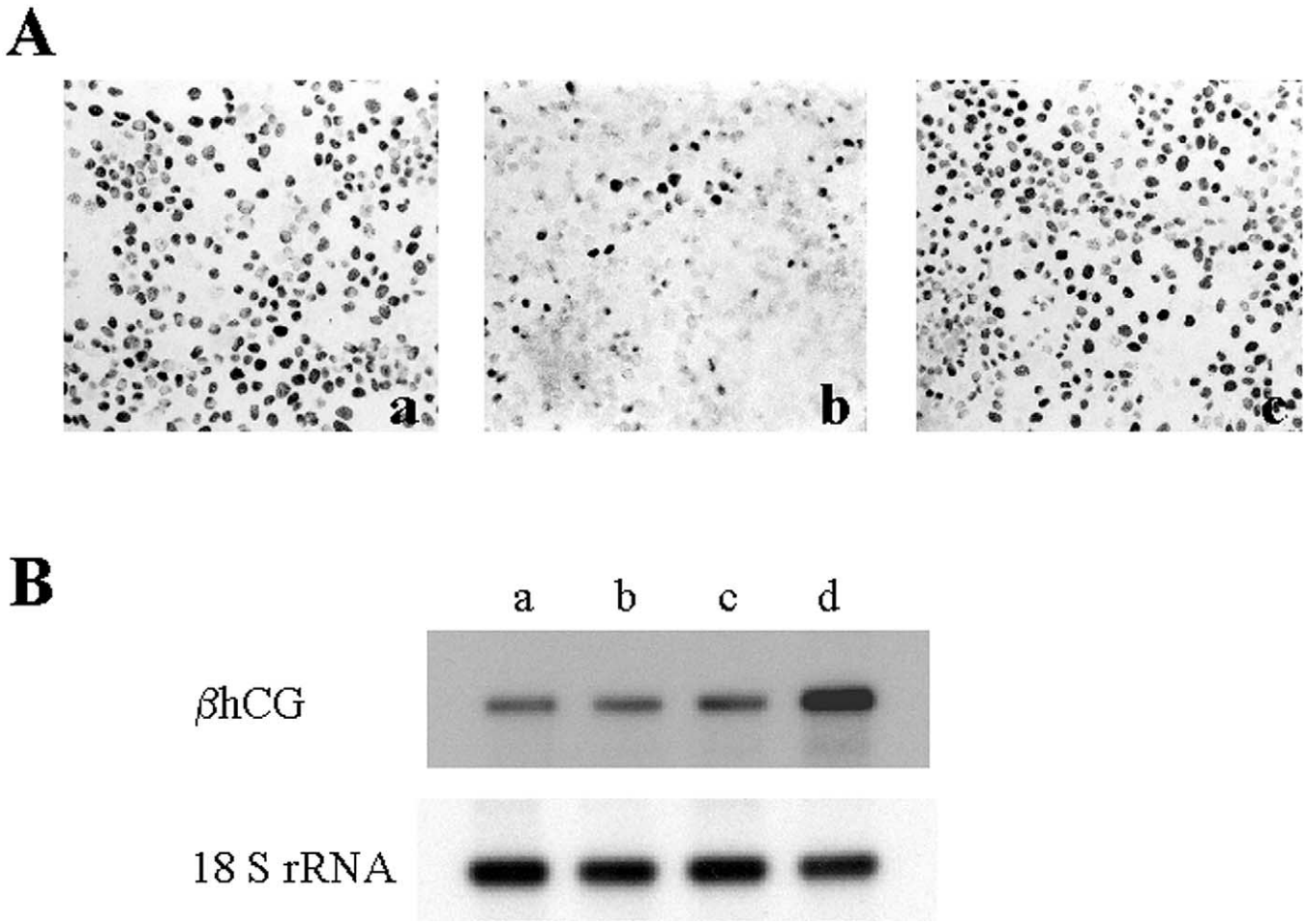
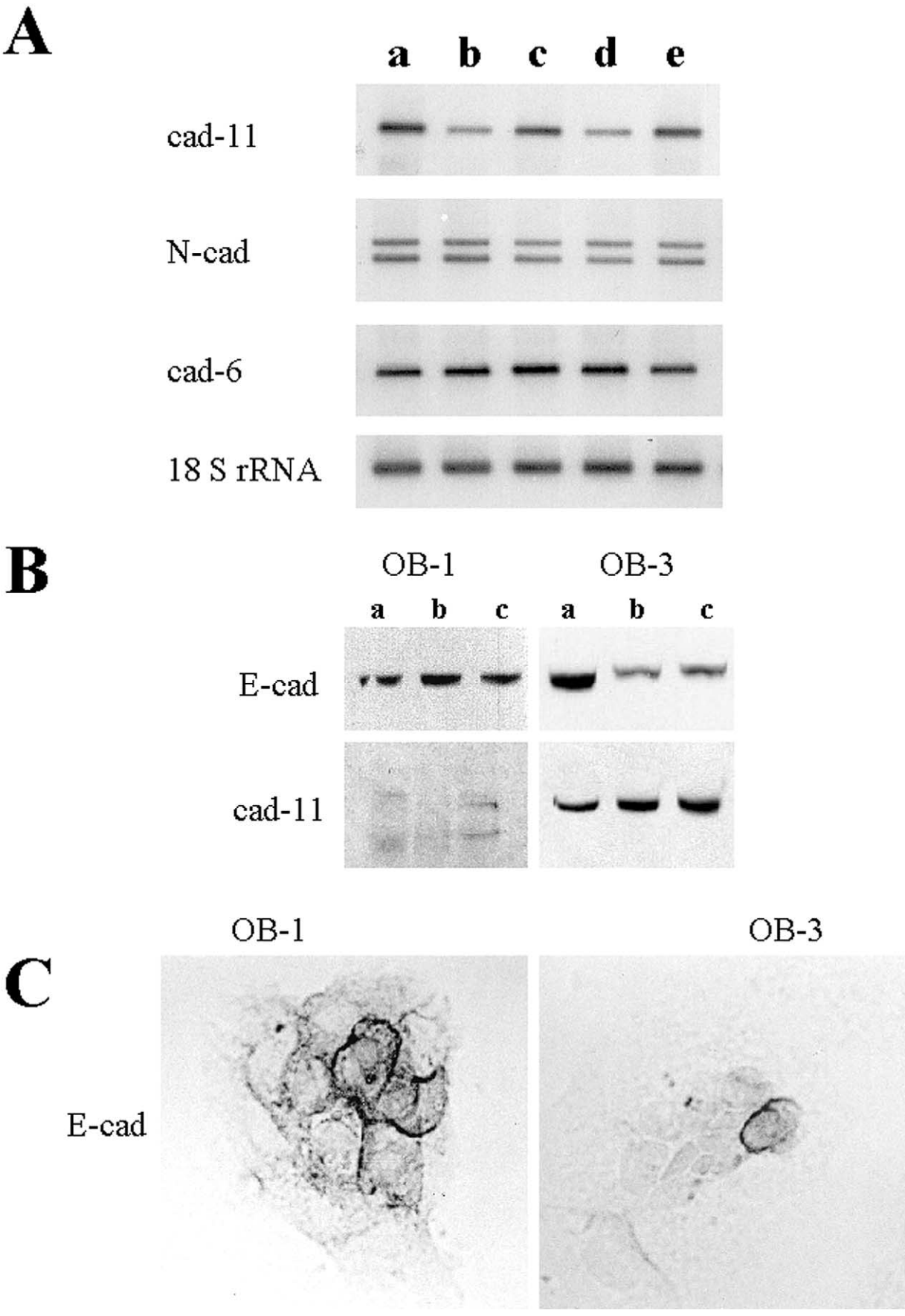


Fig. 6. Ectopic cad-11 expression promotes JEG-3 cell differentiation. (A) Cad-11 reduces cell proliferation in JEG-3 cells. Photomicrographs of BrdU incorporation in JEG-3 cells transfected with p*lac*Z, pCAD-11, or pRCAD-11 for 24 h (a–c, respectively). The cells were fixed and immunostained with a mouse monoclonal antibody directed against BrdU. (B) Ectopic cad-11 expression increases βhCG mRNA levels in JEG-3 cells. Autoradiograms of a Northern blot containing total RNA (20 μg) extracted from untransfected JEG-3 cells (lane a) or JEG-3 cells transfected with p*lac*Z, pRCAD-11, or pCAD-11 and cultured for a further 36 h (lanes b–d, respectively). This blot was probed for βhCG (upper panel) or 18S rRNA (lower panel).

enous cadherin subtypes present in these different cell types remain poorly defined.

The ectopic expression of both type 1 and type 2 cadherins has been shown to increase β-catenin expression in several cell lines and is believed to result from the stabilization of β-catenin when it is complexed to the exogenous cadherin subtype (Papkoff, 1997; Redfield et al., 1997; Shimoyama et al., 2000). In contrast, the transfection of a full-length cad-11 cDNA into JEG-3 cells was associated with a loss of β-catenin from the surface of these cells. The cellular distribution and expression levels of E-cad and β-catenin in these transfected JEG-3 cell cultures correlate with those previously observed in villous cytotrophoblasts undergoing terminal differentiation and fusion in vitro (Getsios et al., 2000). Furthermore, β-catenin was shown to interact with E-cad, but not cad-11, in these primary cell cultures. In view of these observations, it is tempting to speculate that cad-11 mediates the disassembly and/or downregulation of E-cad/β-catenin complexes in JEG-3 cells, resulting in

Fig. 7. Antisense oligonucleotides specific for cad-11 inhibit the morphological differentiation of villous cytotrophoblasts isolated from the human term placenta. (A) Autoradiograms of a Northern blot containing total RNA (20 μg) extracted from human granulosa-lutein cells cultured in the presence of 0, 1, or 5 μM of the antisense oligonucleotide OB-1 (lanes a, b, and d, respectively) or equal amounts of the corresponding sense oligonucleotide, OB-3 (lanes c and e, respectively), for 24 h. This blot was probed for cad-11, N-cad, cad-6, or 18S rRNA. (B) Autoradiograms of Western blots containing protein (20 μg) extracted from primary villous cytotrophoblasts cultured in the presence of either OB-1 or OB-3 (5 μM) for 24, 48, or 72 h (lanes a–c, respectively). Western blot analysis was performed by using mouse monoclonal antibodies directed against human E-cad or cad-11 (113H). (C) Photomicrographs of E-cad expression in primary villous cytotrophoblasts cultured in the presence of either OB-1 or OB-3 (5 μM) for 72 h. The cytotrophoblasts were fixed and immunostained for human E-cad.


A
a b c d e
cad-11
N-cad
cad-6
18 S rRNA
B
OB-1
OB-3
a b c a b c
E-cad
cad-11
C
OB-1
OB-3
E-cad

the morphological differentiation of mononucleate trophoblastic cells in vitro.

In general, it is believed that the adhesive strength of the cadherins is regulated by the cytoplasmic domains of these CAMs interacting with the catenins (Brieher et al., 1996; Ozawa and Kemler, 1998). Cad-11/β-catenin complexes, which are capable of mediating aggregation and sorting when cad-11 is transfected into fibroblastic L cells, have also been detected in signet-cell carcinomas and invasive breast cancer cell lines (Shibata et al., 1996; Pishvaian et al., 1999). Furthermore, an alternative cad-11 mRNA transcript, which contains a frame shift in the coding sequence, resulting in a truncated protein with a smaller transmembrane domain and a different cytoplasmic domain, has been reported in human cancer cells (Okazaki et al., 1994; Kashima et al., 1999; Kawaguchi et al., 1999). The inability of this truncated form of cad-11 to promote adhesion and aggregation in transfected L cells has been attributed to the loss of the β-catenin binding region in the cytoplasmic domain of this variant cadherin subtype. However, the transfection of a truncated cad-11 cDNA construct lacking the β-catenin binding region into human mammary carcinoma cells has been shown to promote adhesion and the formation of gap junctions in these cell cultures (Braungart et al., 2001). In addition, overexpression of a *Xenopus* cad-11 (Xcad-11) construct lacking the β-catenin binding site promotes adhesion and is subsequently able to disrupt neural crest migration in *Xenopus* embryos in a manner similar to the full-length Xcad-11 (Borchers et al., 2001). Finally, several studies have demonstrated the ability of other cadherin subtypes to mediate homophilic adhesion in the absence of β-catenin binding (Navarro et al., 1995; Kreft et al., 1997; Lee et al., 1998; Ozawa and Kemler, 1998). Taken together, these observations suggest that cad-11 can mediate adhesion independent of β-catenin.

α-, β-, and γ-catenin have been colocalized with E-cad in both murine and human placental tissues (Ohsugi et al., 1996; Getsios et al., 2001). In addition, the formation of E-cad/α-catenin complexes has been shown to be critical for the development of this dynamic tissue (Torres et al., 1997). Although β-catenin is believed to be a key structural component of these cadherin/complexes, the differentiation of the trophectoderm does not appear to be affected in β-catenin null mutant mice (Haegel et al., 1995). The ability of these null-mutant mouse embryos to progress beyond this developmental stage has been attributed to an increase in the expression levels and redistribution of γ-catenin within the epithelial cells forming the trophectoderm. Instead of being restricted to desmosomes, γ-catenin was immunolocalized continuously along the basolateral membrane domain of the trophoblastic cells of these null mutant mice (Fleming et al., 1991; Haegel et al., 1995). Similarly, we have determined that E-cad, α, β-, γ-catenin, and p120^{ctn} are coexpressed in human trophoblastic cells in vitro and in vivo (Getsios et al., 2001). However, a switch from E-cad to cad-11 expression, which results in the formation of multinucleated syncytium from these mononucleate cells, is also concomitant with a marked reduction in the expression levels of α-, β-, γ-catenin and p120^{ctn}. These observations indicate that cad-11/catenin interactions may not be required to promote the terminal differentiation and fusion of human trophoblastic cells. To date, the molecular mechanism(s) by which cad-11 mediates these cellular events remain to be elucidated.

Previous studies have indicated that the cellular mechanisms that regulate the biochemical differentiation of human trophoblastic cells are distinct from those involved in the formation of multinucleated syncytium in vitro (Kao et al., 1992). For example, the intracellular secondary messenger, cAMP, is capable of increasing βhCG mRNA levels in JEG-3 choriocarcinoma cells (Burnside et al., 1985) but does not promote the formation of multinucleated syncytium in these cultured cells (Coutifaris et al., 1991; MacCalman et al., 1996). Similarly, the transfection of a full-length connexin-26 cDNA into JEG-3 cells resulted in a decrease in cellular proliferation and a concomitant increase in βhCG secretion with time in culture (Hellmann et al., 1999). However, large multinucleated syncytial structures were not observed in these transfected cell cultures. In contrast, cad-11 was not only capable of promoting the morphological differentiation of JEG-3 cells but reduced cellular proliferation and increased the levels of the βhCG mRNA transcript present in these cultures.

In summary, we have determined that cad-11 is capable of mediating the morphological and functional differentiation of human cytotrophoblasts in vitro. Although the cellular mechanism(s) by which cad-11 promotes the formation of multinucleated syncytium remain poorly understood, our findings suggest that this developmental process involves a coordinated downregulation in the expression levels of E-cad and the cadherin-associated proteins, α-, β-, γ-catenin, and p120^{ctn}, in mononucleate trophoblastic cells.

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